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Herculin, a fourth member of the *MyoD* family of myogenic regulatory genes

(Myf-5/myogenin/MYC/muscle development)

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ABSTRACT **We have identified and cloned herculin, a fourth mouse muscle regulatory gene. Comparison of its DNA and deduced amino acid sequences with those of the three known myogenic genes (*MyoD*, myogenin, and *Myf-5*) reveals scattered short spans with similarity to one or more of these genes and a long span with strong similarity to all three. This long span includes a sequence motif that is also present in proteins of the myc, achaete–scute, and immunoglobulin enhancer-binding families. The herculin gene is physically linked to the *Myf-5* gene on the chromosome; only 8.5 kilobases separate their translational start sites. A putative 27-kDa protein is encoded by three exons contained within a 1.7-kilobase fragment of the herculin gene. When expressed under the control of the simian virus 40 early promoter, transfected herculin renders murine NIH 3T3 and C3H/10T½ fibroblasts myogenic. In doing so, it also activates expression of myogenin, *MyoD*, and endogenous herculin in NIH 3T3 recipients. In adult mice, herculin is expressed in skeletal muscle but is absent from smooth muscle, cardiac muscle, and all nonmuscle tissues assayed. Direct comparison of the four known myogenic regulators in adult muscle showed that herculin is expressed at a significantly higher level than is any of the others. This quantitative dominance suggests an important role in the establishment or maintenance of adult skeletal muscle.**

Cell lineage studies have led to a description of myogenesis as a stepwise developmental progression beginning with a multipotential mesodermal stem cell and ending in a terminally differentiated, multinucleated myotube. The first step in this process produces from a multipotential precursor cell a determined myoblast that still possesses the ability to proliferate but is now committed to the myogenic pathway. A second distinct step involves withdrawal of the myoblast from the cell cycle and activation of a battery of muscle-specific genes, such as those encoding contractile proteins and metabolic enzymes that identify the cell as a myocyte. Subsequent maturation into functional skeletal muscle includes fusion to form multinucleated myotubes of several distinct types.

A crucial step in understanding the regulation of this developmental pathway was the cloning by Davis *et al.* (1) of MyoD cDNA, which upon transfection converts a variety of nonmyogenic cells into functional myoblasts (2). Yet *MyoD* has turned out to be but one member of a family of myogenic genes, which also includes myogenin (3, 4) and *Myf-5* (5). Each can initiate myogenesis in a transfection assay, and their products share one very similar protein sequence that is also characteristic of a larger, extended family of nuclear proteins that includes the myc family, *Drosophila* achaete–scute complex products and immunoglobulin enhancer-binding proteins (1, 6). Some of these have been shown to be sequence-specific DNA-binding proteins, and the shared protein motif is essential for binding (7). MyoD, for example, binds to the mouse muscle creatine kinase enhancer *in vitro* (8).

While all skeletal muscle is thought to develop by the progression outlined above, the types of differentiated cells produced are phenotypically diverse. For example, several subtypes of fast- and slow-contracting fibers exist in fetal and adult skeletal muscle, and this diversity is thought to be a product of developmental history and, in some instances, function (reviewed in ref. 9). These skeletal muscle types express partially overlapping but distinct sets of muscle-specific gene products, implying the existence of a correspondingly diverse and subtle system of muscle gene regulation. This may be provided, at least in part, by different myogenic regulators of the MyoD type acting alone or in specific combinations. In this work we report the cloning and characterization* of herculin, a fourth member of the myogenic regulatory family. Among the presently known myogenic regulatory factors, it is the quantitatively dominant species in adult muscle, suggesting a major role in differentiation and/or maturation of adult skeletal muscle. Its capacity to activate expression of some other members of the family in cell culture experiments is shown, and implications of these results for regulatory pathways *in vivo* are discussed.

MATERIALS AND METHODS

Identification of Mouse *Myf-5*-Positive Plaques. The BALB/c spleen Charon 4A mouse library was a gift from R. Perlmutter and L. Hood (California Institute of Technology). Plaques were transferred to Hybond-N nylon circles (Amersham) and hybridized to human Myf-5 cDNA probe (a gift from H. Arnold, University of Hamburg Medical School) at 42°C overnight in a solution containing 40% formamide, 6× SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate (pH 6.5), 0.5% $NaDodSO_4$, and 50 μg of salmon sperm DNA per ml.

DNA Sequencing. After restriction mapping, fragments of phage insert DNA were subcloned into Bluescript II KS+ (Stratagene) by standard methods (10) and sequenced with a Sequenase DNA sequencing kit (United States Biochemical) with T3 (Stratagene) and T7 (Promega) promoter primers.

Cell Culture and Analysis. NIH 3T3 cells were grown in 90% (vol/vol) Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) calf serum (both GIBCO). C3H/10T½ cells were grown in 90% DMEM/10% (vol/vol) fetal bovine serum (Hazleton Research Products, Lenexa, KS). Cells on 10-cm plates were transfected by a standard calcium phosphate coprecipitation with 5 or 10 μg of the herculin expression vector pSVhrc, 0.5 μg of pY3, which provides resistance to hygromycin B (11), and enough non-

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*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M30499).

specific plasmid or L-cell DNA to make 15 μg. After 12 hr of exposure to DNA, cells were fed fresh medium. After another 24 hr, the medium was changed to DMEM containing 200 μg of hygromycin B (Calbiochem) per ml and one of the following: 10% calf serum, 2.5% fetal bovine serum (NIH 3T3 cells), or 15% fetal bovine serum (C3H/10T½ cells) to select stable transformants. Alternatively, to induce myogenesis in a transient assay for myogenic activity, the medium was changed to DMEM containing 2% horse serum (Flow Laboratories) and 2 μg of insulin (GIBCO) per ml for 3 days, after which the cells were fixed and immunocytochemically stained for myosin heavy chain (as in ref. 1) with Amersham reagents.

RNA Isolation and Analysis. RNAs were prepared by the method of Chomczynski and Sacchi (12). Riboprobes were synthesized from Bluescript I- or II-based templates. Hybridizations for RNase protections were done in 30 μl of 80% deionized formamide/40 mM Pipes, pH 6.4/1 mM EDTA/0.4 M NaCl under mineral oil at 50°C for 12 hr. Unhybridized probes were digested with 300 units of RNase T1 (BRL) at 30°C for 30 min.

RESULTS AND DISCUSSION

A Phage Containing *Myf-5* Also Contains the Related Herculin Gene. To isolate a fragment of the mouse *Myf-5* gene, we screened a BALB/c spleen genomic library at reduced stringency with the 750 base pair (bp) *Pst* I–*Pvu* II fragment of human Myf-5 cDNA (5). This fragment, which does not contain the *myc* homology region of *Myf-5*, was chosen to avoid cross-reaction with *MyoD* and myogenin. Six reproducibly positive plaques were identified, and restriction analyses of their inserts indicated there were two distinct clones among them that represented both possible orientations of the same 14.5-kilobase (kb) genomic insert. More detailed restriction mapping and Southern DNA transfer analyses led to the map of the phage insert shown in Fig. 1. The analysis also revealed two separate *myc* homologies approximately 8 kb apart, suggesting the presence of two different genes. The DNA sequence within and adjacent to both *myc* homologies was determined, and it confirmed that one was indeed part of the mouse *Myf-5* gene and that the other, 8.5 kb upstream, was part of a new but related gene, which we have named "herculin." Their restriction maps are also shown in Fig. 1. The fact that two independent clones with identical insert maps were isolated suggested a true linkage, not linkage by cloning artifact. To confirm linkage in the genome, Southern blots of *Kpn* I-digested mouse genomic DNA were probed with two gene-specific fragments (see the legend to Fig. 1) either separately or together. From the phage map, *Kpn* I is expected to produce a 9-kb chromosomal segment containing both fragments, and in all three cases the probes did identify a single 9-kb hybridizing band (data not shown).

Sequence of the Herculin Gene Reveals Regions Similar to *MyoD*, Myogenin, and *Myf-5*. The nucleotide sequence and predicted amino acid sequence of herculin are shown in Fig. 2*A*. The DNA sequence extends from a consensus TATA element located just upstream of the *Sal* I site to the *Xba* I site located 3′ of an in-frame TAA stop codon. Assignments of internal exon boundaries were confirmed by sequence analysis of products derived by polymerase chain reaction amplification of herculin cDNA from skeletal muscle (which expresses herculin—see below).

The initial basis for proposing the putative 27-kDa protein to be a member of the MyoD family is its strong identity with MyoD, myogenin, and Myf-5 over the 57 amino acid span boxed in Fig. 2*A* and reproduced for comparisons in Fig. 2*B*. Similar domains are found in proteins of the MYC, *Drosophila* achaete-scute, and immunoglobulin enhancer-binding families and include the proposed helix–loop–helix motif (6), which is known to be important for protein dimerization and DNA binding (7). Within this region the herculin protein is most identical to the myogenic regulators myogenin (82%), MyoD (81%), and Myf-5 (79%). MyoD and Myf-5 are the most closely related pair in the family, having 89% identity in this region. By contrast, the relatedness of herculin to nonmyogenic molecules such as c-myc and E12 is only 33% and 36%, respectively.

In addition to the similarity delineated by the box, there are other amino acids conserved among all four myogenic regulators, the majority of which are in a region just upstream of the box, and these are shown as shaded in Fig. 2*A*. There are no other extended regions of amino acid similarity shared by all four molecules, but there are many scattered spans of homology shared by herculin and only one or two of the other myogenic molecules, the most unusual being a stretch between amino acid residues 214 and 229. In this region, the herculin protein sequence is similar to MyoD and Myf-5 but not to myogenin. However, the corresponding nucleic acid sequence reveals a striking homology shared by all four. This can be explained by a difference in reading frame caused by the apparent deletion of a base from myogenin relative to the other three. The surprising apparent conservation of 48 nucleotides of myogenin DNA following a frameshifting event may suggest that this was a relatively recent occurrence or that there is some independent selective pressure for conservation of this sequence in DNA or in RNA.

Expression of Herculin Is Confined to Skeletal Muscle in an Adult Mouse. Fig. 3 shows the result of a RNase protection experiment designed to detect herculin transcripts in the indicated adult mouse tissues. By this sensitive assay, expression of herculin was confined to skeletal muscle; no transcripts were detected in smooth muscle or cardiac muscle or in other tissues. This pattern is not surprising, since MyoD and myogenin are also exclusively expressed in skeletal muscle. However, *Myf-5*, the nearby chromosomal neighbor of the herculin gene, is reported to be expressed in both skeletal and smooth muscle (5). This indicates that despite their proximity in the genome, the genes for herculin and Myf-5 must be subject to independent regulation, at least in the context of smooth muscle. However, coregulation in

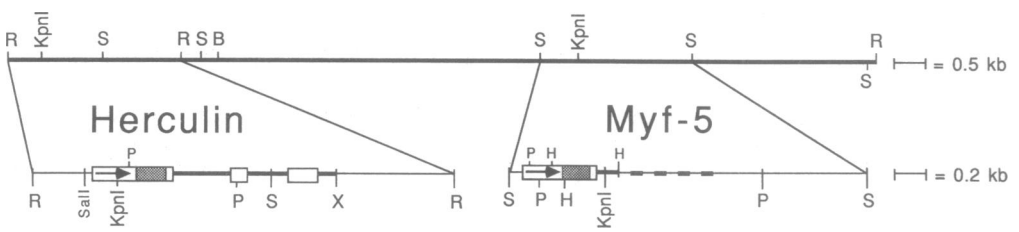

FIG. 1. Phage and plasmid maps showing the genomic organization of herculin and *Myf-5* genes. Boxes identify exons, shading indicates the shared *myc* sequence similarity, and arrows indicate direction of transcription. Only the first exon and part of the first intron of *Myf-5* have been mapped and sequenced; the dotted line represents a portion of the gene not yet analyzed. B, *Bam*HI; H, *Hin*dIII; P, *Pst* I; R, *Eco*RI; S, *Sac* I; X, *Xba* I. Southern blot probes were the *Pst* I–*Sac* I fragment of the herculin gene and the *Sac* I–*Hin*dIII fragment of *Myf-5*.

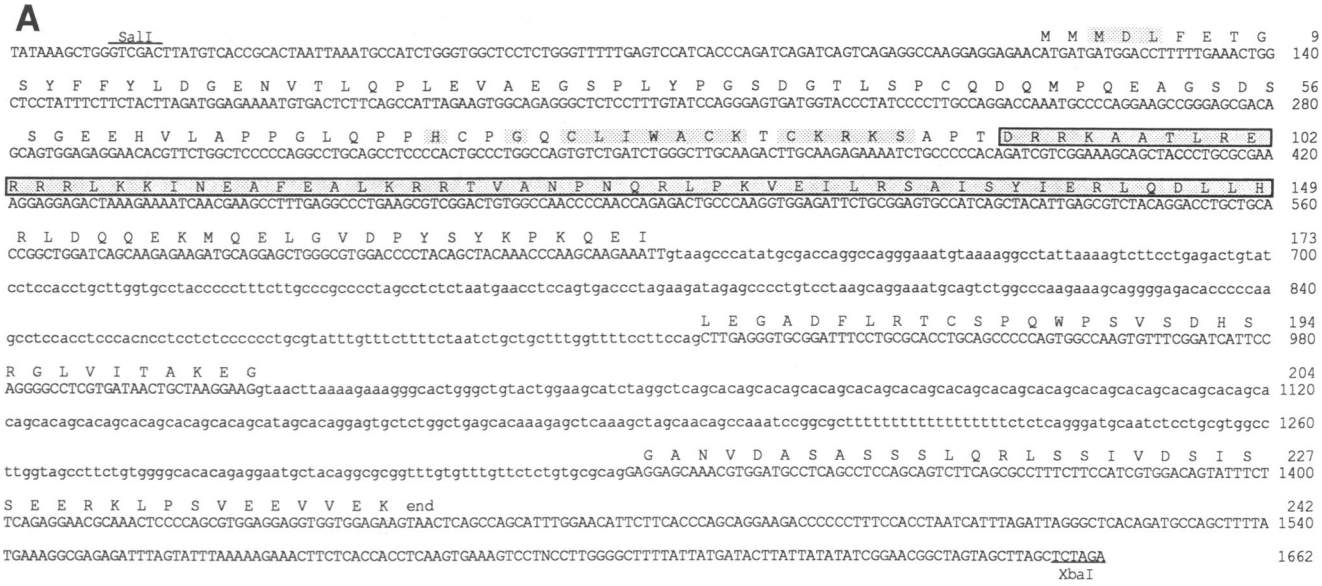

FIG. 2. Nucleotide sequence of the herculin gene and the deduced amino acid sequence of herculin (*A*) and comparison of the myc similarity motifs from the indicated proteins (*B*). The boxed amino acids in *A* comprise this shared motif, and shading outside the box denotes other amino acid spans common to all four proteins. Stars in *B* denote amino acid positions with the most variability among the molecules in this region.

skeletal muscle by one or more shared cis-acting elements is possible, since both genes are expressed there.

Herculin Is Myogenic in NIH 3T3 and C3H/10T½ Cell Backgrounds. To determine whether the sequence similarities between herculin and the other MyoD-related molecules reflect functional similarities, the 1.7-kb *Sal* I–*Xba* I fragment of the herculin gene, which contains the entire putative coding region, was inserted into the expression vector pECE (13). pECE contains a polylinker flanked by the simian virus 40 early promoter and the tumor antigen polyadenylylation signal sequence. The resulting plasmid, pSVhrc, was transfected by calcium phosphate coprecipitation into NIH 3T3 and C3H/10T½ cells together with a selectable marker. These host cells are not myogenic prior to transfection, but herculin expression was found to induce myogenesis in both lines. This was demonstrated in a transient assay by the appearance of myosin heavy chain in individual cells detected by immunostaining (data not shown). The efficiency with which the herculin vector induced myogenic cells was approximately the same as that of an analogous MyoD vector in a parallel experiment. Transfection with the pECE vector alone did not produce any myogenic cells.

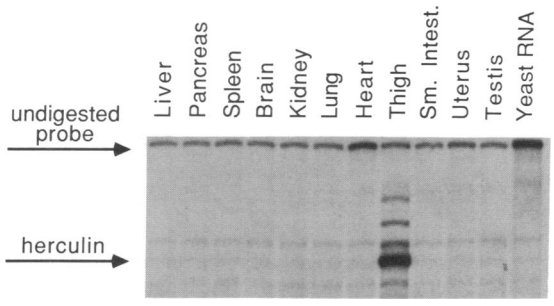

FIG. 3. RNase protection analysis of herculin gene expression in adult mouse tissues. Ten micrograms of total RNA from each tissue was hybridized to an antisense herculin riboprobe extending from nucleotide 321 (*Pst* I) to 12 (*Sal* I).

An interesting and potentially important property of MyoD is that it can, in some recipient cells, activate expression of endogenous *MyoD*, demonstrating a positive autoregulatory pathway (14). We asked whether cells stably transfected with herculin similarly activated the endogenous herculin gene or any of the other cloned myogenic regulatory genes. Stable herculin-transfected NIH 3T3 cells were pooled and cultured under conditions that promoted either proliferation (12.5% serum) or differentiation (2% serum for 3 days). Total cellular RNA was extracted and analyzed by RNase protection assays for expression of herculin, myogenin, and *MyoD* (Fig. 4). Myogenin was activated by herculin but only under differentiation conditions; this is a pattern consistent with myogenin's behavior in other myogenic cell lines (Fig. 4*B*), where it is always expressed upon differentiation. The MyoD and herculin probes are designed to distinguish between endogenous transcripts and those derived from the transfected constructs. The data in Fig. 4*C* demonstrate that exogenous herculin activates endogenous *MyoD* in NIH 3T3 cells, and this activation is most pronounced in cells that have differentiated. The results in Fig. 4*A* show that herculin transformation of NIH 3T3 cells can also activate expression of the endogenous herculin gene, and preliminary experiments suggest a low-level activation of *Myf-5* in these cells as well (J.H.M., unpublished observations). We conclude that herculin can act as a powerful member of the myogenic regulatory network. Its capacity to activate, either directly or indirectly, *MyoD*, myogenin, endogenous herculin, and *Myf-5* argues for a potentially complex network of interactions involving the MyoD family members. It is clear that host-cell characteristics also play a significant role in defining the

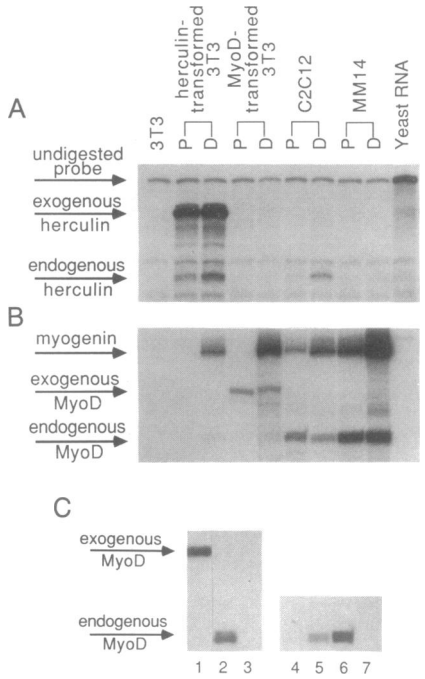

FIG. 4. RNase protection analyses of herculin, myogenin, and *MyoD* gene expression in cell lines under conditions of proliferation (lanes P) or differentiation (lanes D). (*A* and *B*) Separate experiments in which 10 μg of total RNA from each cell line was hybridized to the following riboprobes: herculin, which was the same as in Fig. 3; myogenin, which extended from nucleotide 218 (*Sma* I) to 35 (*Sty* I) (4); MyoD, which extended from an extragenic *Bam*HI site in the MyoD expression vehicle pEMc11s to nucleotide 1692 (*Sty* I) of MyoD cDNA (1). The low level of myogenin expression in proliferating C2C12 and MM14 populations is probably due to a small number of spontaneously differentiated cells. (*C*) Results of additional studies with the MyoD riboprobe alone. Lanes: 1, differentiated MyoD-transformed NIH 3T3 cells; 2, differentiated aza-myoblasts; 3, yeast RNA; 4, proliferating herculin-transformed NIH 3T3 cells; 5, differentiated herculin-transformed NIH 3T3 cells; 6, differentiated C2C12 cells; and 7, yeast RNA. C2C12 cells were grown in 80% DMEM/20% fetal bovine serum and differentiated in 98% DMEM/2% horse serum/2 μg of insulin per ml for 2 days. MM14 cells were grown and differentiated as in ref. 15.

network. In the NIH 3T3 cells studied here, MyoD transfection activates myogenin expression but fails to activate herculin (Fig. 4*A*), *Myf-5* (data not shown) or endogenous *MyoD* (Fig. 4*C*). By contrast, in C3H/10T½ cells, exogenous MyoD does activate its endogenous counterpart (14). Therefore, we expect that host-cell properties are influencing herculin regulatory activity in our cell culture experiments and, very likely, play a significant role in the developing animal as well. Although the importance of host-cell factors cautions against a facile generalization from cell culture model systems to myogenesis *in vivo*, the potent activity of the herculin gene in transfection studies does reveal a circuit for extensive cross-talk between herculin and other family members. This circuitry may prove to be important *in vivo* for initiating a developmental decision or for reinforcing one, once made.

A variety of other muscle cell lines was also examined, and C2C12 (16) was found to be the only one that, under our culture conditions, expressed herculin at detectable levels. MM14 cells (17) and aza-myoblasts (derived by 5-azacytidine treatment of C3H/10T½ cells) (1) did not express herculin (Fig. 4*A* and data not shown). Thus, some independently derived lines can differentiate without detectable expression of herculin, demonstrating that it is neither essential for maintenance of the myoblast phenotype nor for differentiation into muscle. Also, it is notable that in this range of cell types, expression of MyoD or myogenin has not activated expression of herculin. It is not known, however, if any of these lines expressed herculin at some earlier point in their developmental history. The tissue explant-derived line that does express herculin, C2C12, also expresses all of the other family members, and in this respect is similar to herculin-transfected NIH 3T3 cells. Other lines examined express, in the differentiated state, various pairwise combinations of myogenin with only one other member.

Expression of Herculin *in Vivo* Is High Compared with MyoD, Myogenin, and Myf-5. To begin to compare herculin expression in the animal with its activity in cell culture, we measured the relative levels of expression of muscle regulatory genes in adult skeletal muscle by RNase protection assays (Fig. 5) using riboprobes for myogenin and Myf-5 (lane 1), herculin (lane 2), and MyoD (lane 3). Probes were synthesized such that the nuclease-protected probe fragments for each of the four had identical specific activities on a molar basis. Thus, the intensity of each band is proportional to its relative abundance in total RNA from adult skeletal muscle. It is evident that herculin RNA is by far the most prevalent.

Because herculin is the most abundant of the four known myogenic molecules in this tissue, it seems likely that it plays a significant role in the maintenance of the skeletal muscle phenotype in adults. However, it remains to be determined whether herculin is globally expressed in all mature adult skeletal muscle or is restricted to a specific subset of muscle cell types or physiological states. The possibility that herculin is differentially expressed among different muscle cell populations is part of the more general question concerning the roles played by each MyoD family member in muscle tissue and its developmental precursors. Lineage studies suggest that there are different precursor myoblast populations in developing muscle that give rise to distinct muscle fiber types (9). It seems likely that the ultimate differentiated phenotype of these species of skeletal myocytes may be dictated, at least in part, by their patterns of expression of MyoD, myogenin, Myf-5, herculin, and perhaps Myd (18) and ski (19) during development and upon maturation. A comprehensive picture of the pattern of expression for each of these regulators during the life history of a given myogenic lineage is not presently available, but an initial study of MyoD and myogenin in the developing mouse embryo has found significant diversity among different cell populations. By hybridization to RNA *in situ*, Sassoon *et al.* (20) found at least two different

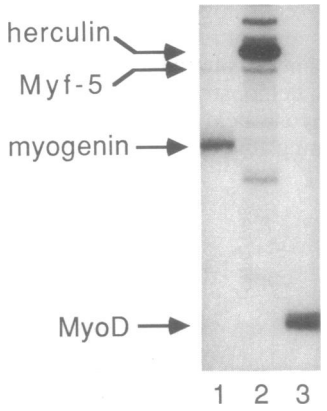

FIG. 5. RNase protection analyses of herculin, Myf-5, myogenin, and MyoD transcripts in adult skeletal (thigh) muscle. Total RNA (10 μg) was hybridized to the following riboprobes: Myf-5 and myogenin (lane 1), herculin (lane 2), and MyoD (lane 3). The Myf-5 probe was synthesized from a 382-bp *Hin*dIII fragment that includes the end of the first exon (see Fig. 1). The other probes were as described in the legends to Figs. 3 and 4.

patterns of expression coincident with two different types of muscle, myotomal and limb. They also reported the presence of determined myoblasts in the limb bud that expressed no detectable MyoD or myogenin, though some of these myoblasts did express detectable levels after 4 or 5 days in culture. Were these myoblasts initially determined by expression of a different myogenic molecule, such as herculin, Myd, or Myf-5, which later activated expression of MyoD and myogenin? Such a temporal sequence of expression of these molecules during development might arise from the circuitry and changing host-cell influences identified in our cell culture experiments. This regulatory history could then lead to a final set of regulators that governs the functional identity of a mature myocyte.

Note Added in Proof. With respect to expression of Myf-5 (5), Arnold and coworkers (21) have continued investigation and recently reported that it is absent from smooth muscle. Therefore, herculin and *Myf-5* (as well as *MyoD* and myogenin) are similar in their restriction of expression to skeletal muscle.

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